Published April 4, 2005

Phenylbutyrate up-regulates the *adrenoleukodystrophy-related* gene as a nonclassical peroxisome proliferator

Catherine Gondcaille,[1] Marianne Depreter,[2] Stéphane Fourcade,[1] Maria Rita Lecca,[1] Sabrina Leclercq,[1] Pascal G.P. Martin,[3] Thierry Pineau,[3] Françoise Cadepond,[4] Martine ElEtr,[4] Nathalie Bertrand,[5] Alain Beley,[5] Sandrine Duclos,[1] Dirk De Craemer,[2] Frank Roels,[2] Stéphane Savary,[1] and Maurice Bugaut[1]

[1]Laboratoire de Biologie Moléculaire et Cellulaire, Faculté des Sciences Gabriel, 21000 Dijon, France
[2]Department of Human Anatomy, Embryology, Histology and Medical Physics, Ghent University, 9000 Gent, Belgium
[3]Laboratoire de Pharmacologie et Toxicologie, Institut National de la Recherche Agronomique, 31931 Toulouse, France
[4]Institut National de la Santé et de la Recherche Médicale U488, 94276 Le Kremlin-Bicêtre, France
[5]Laboratoire de Pharmacodynamie, Faculté de Pharmacie, 21000 Dijon, France

X-linked adrenoleukodystrophy (X-ALD) is a demyelinating disease due to mutations in the *ABCD1* (*ALD*) gene, encoding a peroxisomal ATP-binding cassette transporter (ALDP). Overexpression of adrenoleukodystrophy-related protein, an ALDP homologue encoded by the *ABCD2* (*adrenoleukodystrophy-related*) gene, can compensate for ALDP deficiency. 4-Phenylbutyrate (PBA) has been shown to induce both *ABCD2* expression and peroxisome proliferation in human fibroblasts. We show that peroxisome proliferation with unusual shapes and clusters occurred in liver of PBA-treated rodents in a PPARα-independent way. PBA activated *Abcd2* in cultured glial cells, making PBA a candidate drug for therapy of X-ALD. The *Abcd2* induction observed was partially PPARα independent in hepatocytes and totally independent in fibroblasts. We demonstrate that a GC box and a CCAAT box of the *Abcd2* promoter are the key elements of the PBA-dependent *Abcd2* induction, histone deacetylase (HDAC)1 being recruited by the GC box. Thus, PBA is a nonclassical peroxisome proliferator inducing pleiotropic effects, including effects at the peroxisomal level mainly through HDAC inhibition.

Introduction

X-linked adrenoleukodystrophy (X-ALD; OMIM 300100) is an inherited disorder characterized by progressive demyelination of the central nervous system and adrenal insufficiency (Moser et al., 2001). X-ALD is associated with an accumulation of very long-chain fatty acids (VLCFA) in plasma and tissues. The disease is due to mutations in the *ABCD1* (ALD) gene located in Xq28 (Mosser et al., 1993). *ABCD1* encodes the protein ALDP, a peroxisomal member of the ATP-binding cassette family, which is thought to participate in the entry of VLCFA into the peroxisome where VLCFA are β-oxidized. ALDP is a half-transporter, which is supposed to function as a homodimer or a heterodimer, in association with one of the three other peroxisomal ATP-binding cassette half-transporters, ALDRP (adrenoleukodystrophy-related protein), which is the closest homologue of ALDP (Lombard-Platet et al., 1996), PMP70 (70-kD peroxisomal membrane protein; Kamijo et al., 1990), and PMP69 (Holzinger et al., 1997). These transporters are encoded by the *ABCD2* (*Adrenoleukodystrophy-related*), *ABCD3*, and *ABCD4* genes, respectively, and their function is still unclear. Overexpression of ALDRP has been demonstrated to compensate for ALDP deficiency in *Abcd1* −/− mice, thus preventing VLCFA accumulation and the onset of a neurological phenotype (Pujol et al., 2004). Furthermore, restoration of VLCFA β-oxidation could be obtained in X-ALD human fibroblasts transfected with *Abcd2* cDNA (Braiterman et al., 1998; Kemp et al., 1998; Flavigny et al., 1999; Netik et al., 1999; Fourcade et al., 2001). Therefore, pharmacological induction of this partially redundant gene could be a therapeutic strategy for X-ALD. We have shown that fibrates up-regulate *Abcd2*

Dr. Bugaut died on 16 September 2004.

Correspondence to Stéphane Savary: stsavary@u-bourgogne.fr

M. Depreter's present address is Institute for Stem Cell Research, University of Edinburgh, Edinburgh EH9 3JQ, UK.

Abbreviations used in this paper: AOX, acyl-CoA oxidase; DAPA, DNA affinity precipitation assay; EMSA, electrophoretic mobility shift assay; HAT, histone acetyl transferase; HDAC, histone deacetylase; LM, light microscopy; PBA, 4-phenylbutyrate; PP, peroxisome proliferator; TSA, trichostatin A; VLCFA, very long-chain fatty acids; X-ALD, X-linked adrenoleukodystrophy.

The Journal of Cell Biology, Vol. 169, No. 1, April 11, 2005 93–104
http://www.jcb.org/cgi/doi/10.1083/jcb.200501036

Published April 4, 2005

expression (Albet et al., 1997, 2001; Berger et al., 1999; Fourcade et al., 2001) in the liver of rodents. Fibrates can restore β-oxidation of VLCFA in the liver of *Abcd1* −/− mice (Netik et al., 1999) but not in brain, possibly due to obstacle of the blood–brain barrier (Waddell et al., 1989; Berger et al., 1999). Fibrates, commonly used as hypolipidemic drugs in human medicine, are peroxisome proliferators (PPs) in rodents. PPs are ligands of a member of the steroid nuclear receptor family named PPARα (PP-activated receptor α). PPARα up-regulates expression of target genes involved in lipid metabolism by binding to a DNA sequence called PPRE (PP response element). However, such a functional PPRE has not been found in the *Abcd2* promoter (Gartner et al., 1998; Fourcade et al., 2001).

4-Phenylbutyrate (PBA) treatment can restore β-oxidation of VLCFA and increase *ABCD2* expression in fibroblasts from X-ALD patients and *Abcd1* −/− mice (Kemp et al., 1998). Furthermore, dietary PBA was shown to be efficient in vivo to reduce the VLCFA levels in the brain of *Abcd1* −/− mice, but *Abcd2* expression has not been analyzed (Kemp et al., 1998). Interestingly, the authors observed a 2.4-fold increase in the number of peroxisomes in 5 mM PBA-treated control or X-ALD human fibroblasts, which was not accompanied by induction of the *PPARα* and peroxisomal *Acyl-CoA Oxidase* (*AOX*) genes but could be linked to the observed induction of the *PEX11α* gene. This hypothesis was strengthened by the demonstration that PBA-mediated hepatic peroxisome proliferation is absent in *Pex11α* −/− mice (Li et al., 2002). Classical PPs do not cause peroxisome proliferation in humans, and fibroblasts are not known to be the target cells for PPs. Moreover, both peroxisome proliferation and *PPARα* and *Aox* induction occur in PP-treated rodents. Kemp et al. (1998) reported induction of *PPARα*, but not of *Aox*, in PBA-treated mice fibroblasts. By contrast, activation of a reporter plasmid containing the rat *Aox* PPRE has been obtained in PBA-treated mouse hepatoma cells (Pineau et al., 1996) and the binding of PBA to PPARα has been demonstrated using C6 rat glioma cells (Liu et al., 2002). It should also be noticed that an increased expression of PPARα has been observed in PBA-treated human glioma cells (Pineau et al., 1996). Together, the data indicate that PBA, a compound structurally related to fibrates, is a PP that acts partially through noncanonical mechanisms.

Sodium butyrate induces a variety of alterations at the molecular and cellular levels. Transcriptional activation, as a result of the inhibition of histone deacetylase (HDAC) activity could represent the main mechanism of action of butyrate (Davie, 2003). PBA, a butyrate analogue, displays also similar pleiotropic effects in vitro and in vivo. It has been reported that PBA induces hyperacetylation of histones (Lea and Randolph, 1998; Warrell et al., 1998). Deacetylase inhibition may account for *ABCD2* induction observed in PBA-treated fibroblasts and probably for *PEX11α* induction, which might be responsible for the PPARα-independent peroxisome proliferation.

Our main objective was to compare the effects of PBA, butyrate, and fibrate on the expression of *Abcd2* and *Aox* and peroxisome proliferation in vivo in the liver and brain of rats and in vitro in hepatic and glial cells. The molecular mechanism of the PBA regulation of *Abcd2* expression was also studied.

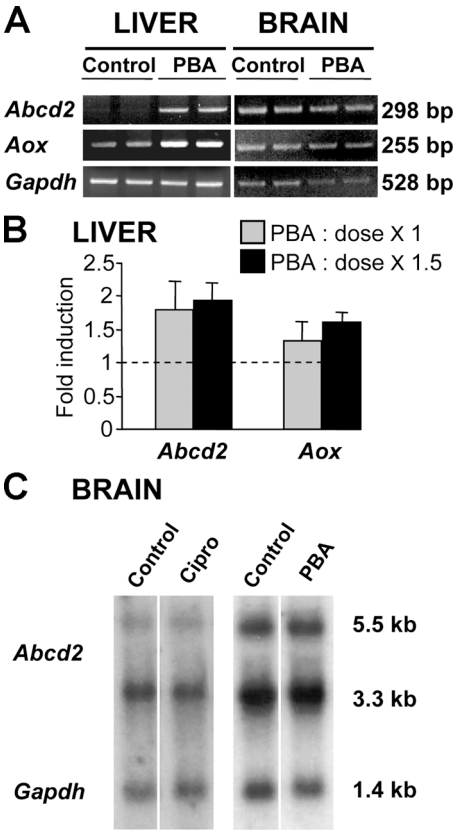

Figure 1. **Expression of the *Abcd2* and *Aox* genes is up-regulated by PBA in liver.** (A) RNA samples from liver and brain of a control rat and from a rat treated per os with PBA (dose ×1.5) for 9 wk were analyzed by RT-PCR in duplicates. (B) RNA was extracted from liver of control rats ($n = 10$) and rats treated with PBA (dose ×1) for 6–7 wk ($n = 6$) or PBA (dose ×1.5) for 6–9 wk ($n = 4$). Samples were individually analyzed twice by RT-PCR. The intensities for *Abcd2* and *Aox* mRNA were quantified by digital imaging and normalized using *glyceraldehyde-phosphate dehydrogenase* (*Gapdh*) intensities. Data (means ± SD) are presented as fold induction in relation to the values for control rats (taken equal to 1 and not depicted in the figure). (C) 2 mg sodium ciprofibrate (Cipro) or 20 mg PBA in 100 μl of water was injected into the cerebral fourth ventricle (100 μl/h). Control rats received equiosmolar amounts of NaCl. RNA was extracted from the whole brain and analyzed individually by Northern blotting ($n = 4$ for each group of rats).

Results

PBA up-regulates expression of the *Abcd2* and *Aox* genes in liver but not in brain

Because the liver is the main target tissue for PPs and demyelination occurs in the brain of X-ALD patients, we evaluated the impact of PBA on peroxisomal gene expression in these tissues. Rats were treated per os with PBA as described by Kemp et al. (1998) (dose ×1) and also with a higher dose (dose ×1.5) during 6–9 wk. Expression of the *Abcd2* and *Aox* genes was increased in the liver (Fig. 1 A), with marked interindividual variations as it was already seen for PPARα (Lemberger et al., 1996). No significant difference could thus be obtained between the two doses (Fig. 1 B). Considering the 10 treated animals, the average induction was 1.9- and 1.5-fold for the *Abcd2* and *Aox* genes, respectively. Gene induction was observed neither in the brain (Fig. 1 A) nor in the spleen, kidney, and testis (unpublished data).

Published April 4, 2005

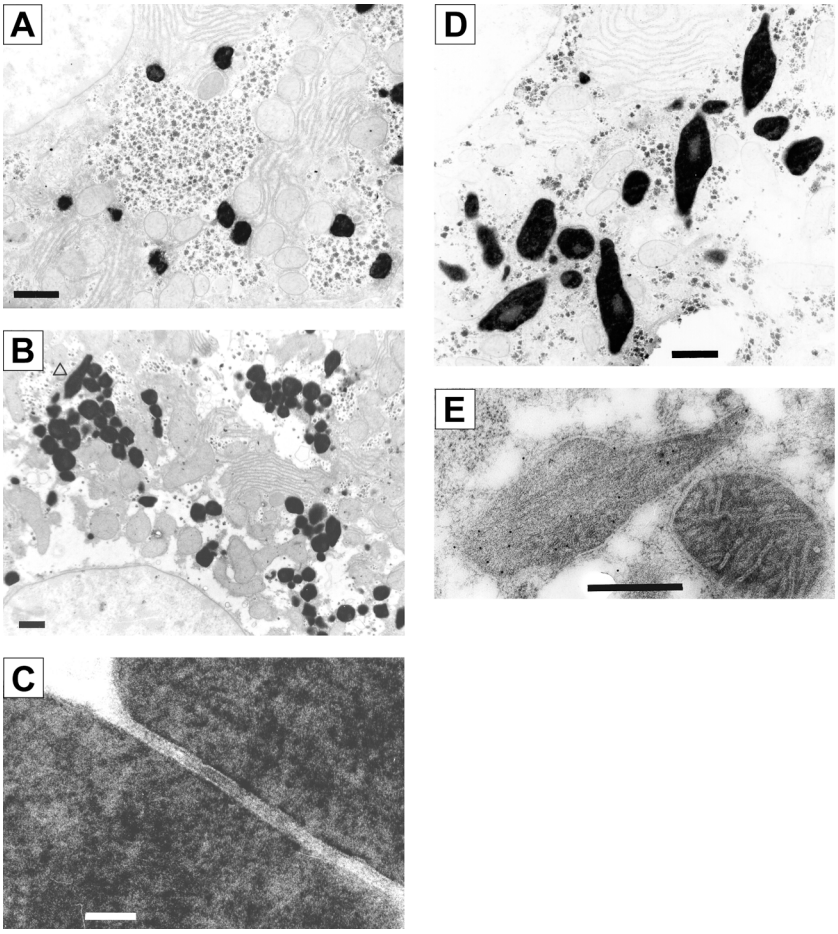

Figure 2. **PBA induces peroxisome proliferation in liver.** (A–D) Peroxisomes were stained for catalase activity. Liver of a control rat (A) and a rat treated per os with PBA (dose ×1.5) for 6 wk (B). Note peroxisome clusters and a phi-shaped peroxisome (arrowhead) in B. (C) Nearly touching peroxisomes suggesting a fission or budding process (Roels, 1991) in liver of a rat treated with PBA (dose ×1) for 6 wk. (D) Four phi-shaped peroxisomes in liver of a rat treated as in C. Unstained nucleoids are clearly visible in several peroxisomes. (E) Immunogold localization of AOX in a phi-shaped peroxisome, adjacent to an unlabeled mitochondrion, in liver of a rat treated as in C. Bars: (A, B, D, and E) 1 μm; (C) 100 nm.

Induction of peroxisomal genes by PPs usually starts several hours after the beginning of a treatment (Motojima, 1997). Because a unique IP injection of PBA (400 mg/kg) was sufficient to weakly induce (1.5-fold) the expression of a PP-sensitive gene (*CYP4A1*; Pineau et al., 1996), we injected 720 mg of PBAper kilogram per day i.p. for 3 d and analyzed mRNA from liver, brain, intestine, muscle, spleen, kidney, and lung. No induction of the *Abcd2* and *Aox* genes was obtained in the seven examined tissues (unpublished data).

The absence of gene induction in the brain after long- and short-term treatments, which could result from a rapid catabolism (the half-life of PBA is ∼1–2 h), prompted us to perfuse PBA directly in the fourth ventricle. No induction of the two peroxisomal genes was found in the brain examined as a whole (Fig. 1 C). In the same way, no change in the gene expression was obtained when ciprofibrate was perfused under the same experimental conditions.

PBA treatment induces peroxisome proliferation in liver along with appearance of peroxisomal phi-bodies and clusters

Peroxisomes were observed in the liver of each control and of each per os PBA-treated rat by light microscopy (LM) and EM. When comparing the pooled data from all the treated rats (n = 10) with the pooled data from all the control rats (n = 10), peroxisome proliferation was clearly demonstrated. LM examination showed that the number of peroxisomes per cellular area was higher in the treated animals than in the control animals (×1.4; $P < 0.05$). By EM morphometric analysis, significantly higher numerical density (×2.3; $P < 0.001$), volume density (×2.3; $P < 0.01$), and surface density (×2.2; $P < 0.01$) versus controls were found in the treated rats, giving evidence of peroxisome proliferation (Fig. 2, A and B). Although the average size of peroxisomes was not significantly different in the control and treated rats, a small percentage of peroxisomes in 8 out of 10 treated animals had very large size (D-circle 2.7-fold that of control peroxisomes) and showed peculiar shapes (phi-bodies; Fig. 2 D). These phi-bodies were present in multiple fragments of the same liver and mostly located in the periportal areas. Phi-bodies were never seen in control livers. Furthermore, clusters of up to 10 peroxisomes were present in the rats treated with dose ×1.5 (Fig. 2 B), whereas in the control rats as well as in the rats treated with dose ×1, only clusters of two to three peroxisomes were seen (peroxisomes are considered to belong to a cluster when the distance between neighboring organelles is smaller than twofold of the mean diameter of the peroxisomes of the cluster). The clusters often showed nearly touching peroxisomes (Fig. 2 C). Finally, heterogeneity in the reaction product of catalase activity was detected in proliferated peroxisomes, which was not observed in control rats. AOX was located in peroxisomes by EM immunodetection (Fig. 2 E). The mean label-

Published April 4, 2005

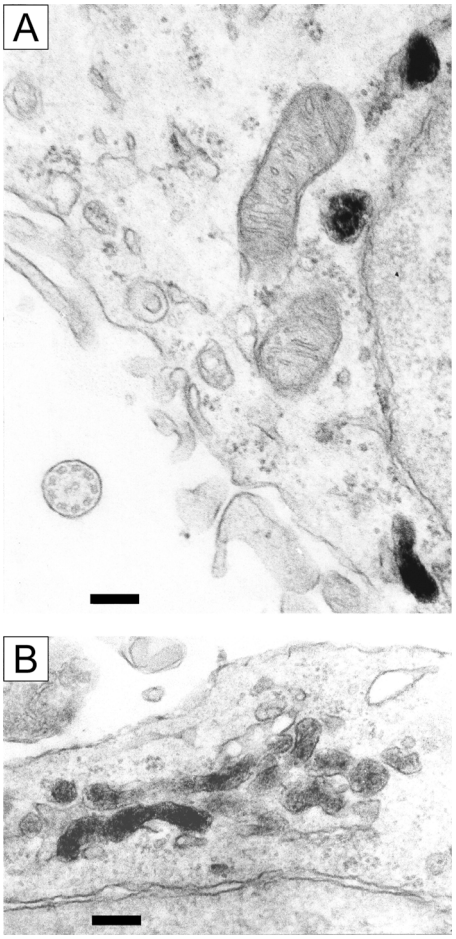

Figure 3. **PBA modifies peroxisome distribution in ependymal cells of the fourth ventricle.** Peroxisomes were stained for catalase activity. Peroxisomes in cells of a control rat (A) or a rat treated per os with dose ×1 of PBA for 6 wk (B). Peroxisomes are small and few when compared with liver (Fig. 2). Peroxisome clusters as shown in B were more frequently observed in treated animals. Cilium is seen in A. Bars, 200 nm.

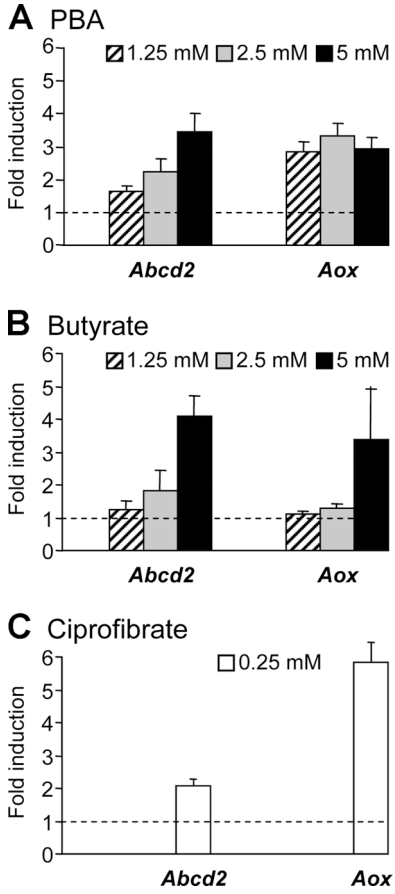

Figure 4. **Peroxisomal genes are inducible by PBA and butyrate in hepatocytes.** Rat primary hepatocytes were cultured in the presence of different concentrations of PBA, butyrate, and ciprofibrate for 72 h. Total RNA was analyzed by RT-PCR for *Abcd2* and *Aox* expression. Results are presented as fold induction in relation to the control values for cells cultured in the absence of drug. Control data, taken equal to 1, are not reported in the figure. Data represent means ± SD of two independent experiments.

ing density for AOX was increased by a factor of 1.3 in PBA-treated rats ($n = 3$) in comparison with control rats ($n = 3$). In addition, when the volume density of the peroxisomes was taken into account, the total labeling differed by a factor 2.5 between the control and treated rats. Although not significant, these differences are in agreement with RT-PCR data (Fig. 1, A and B).

A weak but significant dose effect was observed in the liver of the PBA-fed rats. The number of peroxisomes per cellular area measured by LM increased by 1.4-fold with dose ×1 and 1.7-fold with dose ×1.5. Furthermore, the EM morphometry values were always higher in the liver of rats ($n = 4$) treated with dose ×1.5 (numerical density: ×2.7, volume density: ×3.1, and surface density: ×2.9) than in the rats ($n = 6$) treated with dose ×1 (×2.1, ×1.9, and ×1.8, respectively). A peroxisome proliferation index was calculated for each treated animal ($n = 10$) versus the mean value of 10 control animals considering successively the number of peroxisomes per cellular area measured by LM and each of the three EM morphometry values. When the four proliferation factors of each treated animal were ranked ($n = 40$), the dose effect was significant ($P < 0.001$). In fact, the different animals did not respond to the PBA treatment to the same extent with regard to the number of peroxisomes, as it was observed already for the induction of peroxisomal genes (Fig. 1 B). In some animals, a clear proliferative effect co-occurred with a higher induction of peroxisomal genes.

The number of peroxisomes per cellular area was studied by LM in the kidney (tubulus contortus I cells) and the intestinal epithelium, two PP-sensitive tissues, and showed no significant differences between the control rats ($n = 3$) and the per os treated rats ($n = 3$) with dose ×1 of PBA.

In the rats i.p. injected with PBA during 3 d, LM examination revealed no peroxisome proliferation in the liver, in agreement with the absence of induction of peroxisomal genes as mentioned in the previous paragraph.

PBA treatment modifies the peroxisome distribution in ependymal cells of the fourth ventricle

Peroxisomes were also studied by EM in ependymal cells lining the fourth ventricle of control rats ($n = 7$) and per os PBA-treated rats with doses ×1 ($n = 3$) or ×1.5 ($n = 3$). A 1.4-fold ($P = 0.05$) increase in the number of peroxisomes per cytoplas-

Published April 4, 2005

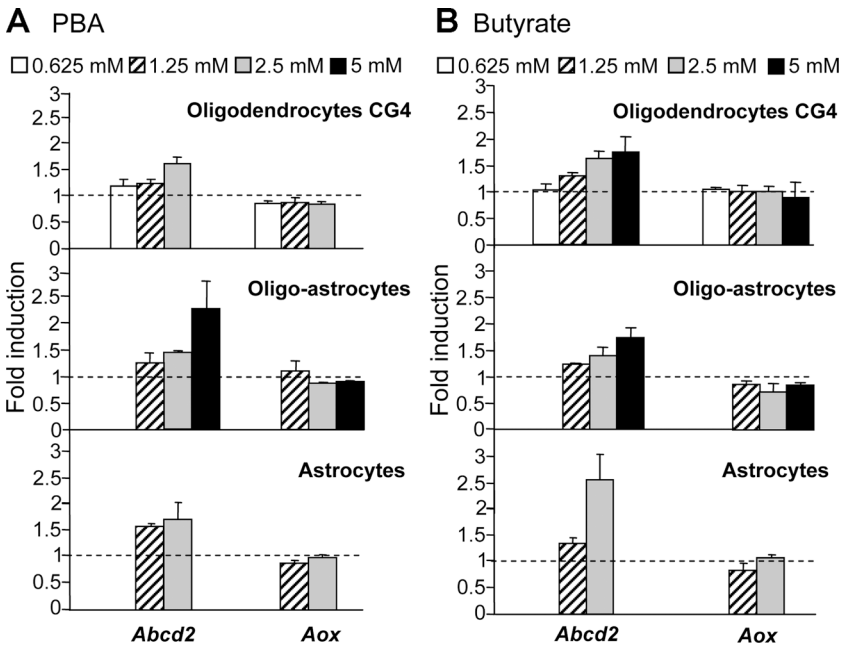

Figure 5. ***Abcd2* is inducible by PBA and butyrate in glial cells.** Differentiated CG-4 oligodendrocytes, pure primary astrocytes, and mixed primary cultures of oligodendrocytes and astrocytes were treated with different concentrations of PBA (A) and butyrate (B) for 72 h. Gene expression was analyzed by RT-PCR. Results are means ± SD of three independent experiments and presented as in Fig. 4.

mic area occurred together with more frequent clusters (×2.0; $P < 0.05$) (Fig. 3). However, EM revealed no difference in ependymal cells of the choroid plexus or lining the lateral wall of the lateral ventricle and in stem cells of the subventricular zone. These stem cells contained only very few and small catalase particles.

When PBA was infused directly into the fourth ventricle, no effect on peroxisomes was observed by EM in ependymal cells, whereas infusion of ciprofibrate caused a decrease (×0.67; $P < 0.05$) in the number of peroxisomes per cell profile.

PBA and butyrate induce expression of peroxisomal genes and proliferation of peroxisomes in primary cultures of hepatocytes

Considering the short half-life of butyrate in living animals (~6 min), primary cultures of rat hepatocytes were treated with butyrate, PBA, and ciprofibrate for 72 h to gain insight into the mechanisms involved in the regulation of gene expression and peroxisome biogenesis. PBA and butyrate displayed a similar dose-dependent activation of *Abcd2* expression (Fig. 4). However, the increase in *Aox* mRNA levels was lower after butyrate treatment than after PBA treatment (Fig. 4). Furthermore, the *Abcd2* expression level relative to *Aox* level was higher with PBA and butyrate than with ciprofibrate (Fig. 4). Therefore, the effects on peroxisomal gene expression in primary hepatocytes appeared to be different for the three compounds.

Catalase-stained peroxisomes were visualized in hepatocytes by LM. The number of peroxisomes per cellular area was increased 1.4-fold ($P < 0.01$) in PBA-treated cells versus controls for dose 1.25 mM and 1.8-fold ($P < 0.001$) for dose 2.5 mM. An increase was also seen when cells were treated with butyrate: 1.3-fold ($P < 0.05$) for dose 1.25 mM and 1.4-fold ($P < 0.01$) for dose 2.5 mM. A similar increase (×1.7; $P < 0.001$) was obtained after 0.25 mM ciprofibrate treatment.

PBA and butyrate, but not ciprofibrate, induce *Abcd2* expression and peroxisome proliferation in cultured glial cells

In the prospect of a possible pharmacological therapy of X-ALD, it is relevant to try to induce *Abcd2* expression in glial cells. In differentiated CG4 cells, pure primary astrocytes, and mixed primary cultures of glial cells, we observed a dose-dependent induction of *Abcd2* expression after PBA or butyrate treatment but no effect on *Aox* expression (Fig. 5, A and B). When the glial cells were treated with 0.25 and 0.5 mM ciprofibrate, we observed no change in the *Abcd2* and *Aox* expression in the three types of cell culture (unpublished data).

We also examined the peroxisomes by LM after immunolocalization of catalase in C6 rat glioma cells treated with 5 mM PBA or 0.5 mM ciprofibrate. The number of peroxisomes per cellular area was significantly higher in PBA-treated cells than in controls (×2.0; $P < 0.01$) but remained unchanged in ciprofibrate-treated cells. The PBA treatment generated a change in cell morphology, characterized by a fibroblastic aspect, which was much less marked when cultures were treated with ciprofibrate (unpublished data).

PBA induction of the *Abcd2* gene and peroxisome proliferation does not require PPARα

We have previously shown that fenofibrate induction of *Abcd2* and *Aox* in the liver of rodents requires the presence of PPARα (Fourcade et al., 2001). To test whether or not PBA induction of the peroxisomal genes is dependent on PPARα, fibroblasts from control and *PPARα* −/− mice were treated with PBA or fenofibrate and mRNA was analyzed. In contrast to hepatocytes (Fig. 4), the peroxisomal genes in control fibroblasts were not induced by fenofibrate (Fig. 6 A), as observed in glial cells (unpublished data). PBA elicited the same dose-dependent induction of *Abcd2* in both *PPARα* +/+ and *PPARα* −/−

Published April 4, 2005

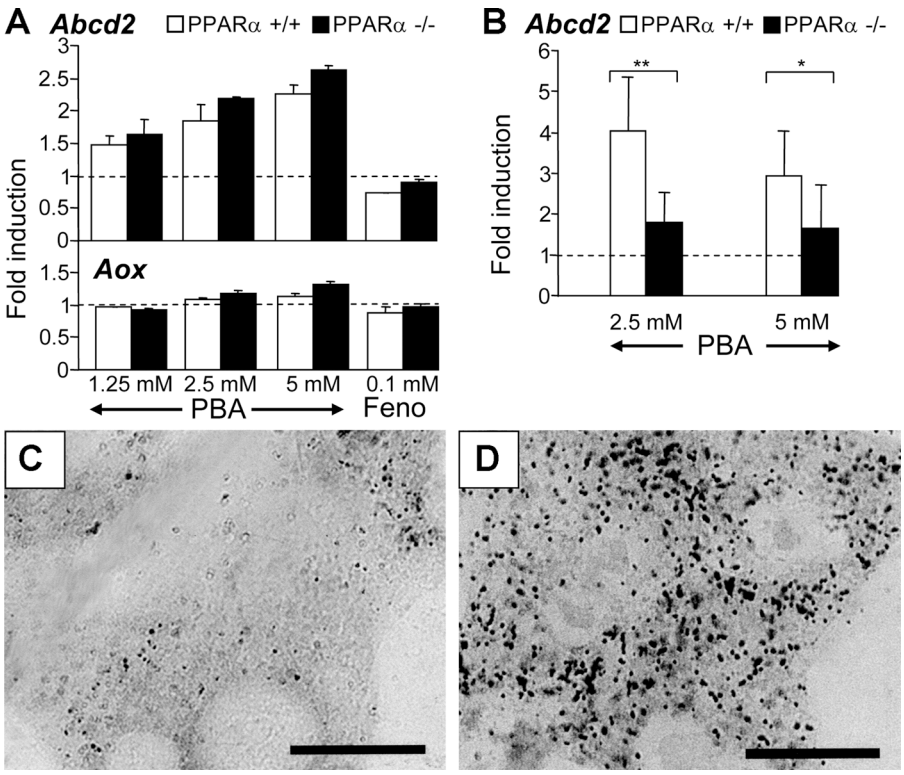

Figure 6. **PBA causes *Abcd2* induction and peroxisome proliferation in PPARα −/− cells.** Fibroblasts (A) and hepatocytes (B) were prepared from fetal and adult liver, respectively, of *PPARα* +/+ and *PPARα* −/− mice and exposed to PBA or fenofibrate (Feno) for 72 h. Gene expression was analyzed by RT-PCR. Data are means ± SD of two to five independent experiments and presented as in Fig. 4. Untreated (C) or 2.5 mM PBA-treated (D) hepatocytes from *PPARα* −/− mice were examined for the number of peroxisomes by LM after anti-catalase protein A–gold immunolabeling with silver enhancement. Bars, 20 μm.

fibroblasts, indicating that it does not require PPARα to activate *Abcd2* (Fig. 6 A). The changes in expression of *Aox* in the PBA-treated fibroblasts were too weak (as already observed in PBA-treated normal fibroblasts by Kemp et al. [1998]) to conclude whether or not their induction by PBA is PPARα dependent (Fig. 6 A). Similar results were obtained using hepatocytes, except that PBA induction of *Abcd2* was significantly higher in *PPARα* +/+ cells than in *PPARα* −/− cells (Fig. 6 B), indicating that the extra induction of *Abcd2* in normal hepatocytes is PPARα dependent. Furthermore, the peroxisome proliferation induced by PBA in control hepatocytes (see the section PBA and butyrate induce expression of peroxisomal genes and proliferation of peroxisomes in primary cultures of hepatocytes) was maintained in *PPARα* −/− hepatocytes (Fig. 6, C and D) as the peroxisome number measured by LM was increased in cells treated with 2.5 (×1.7; $P < 0.05$) and 5.0 mM PBA (×1.4; $P < 0.05$). This result was confirmed using EM (×2.7 in 5.0 mM PBA-treated cells).

Trichostatin A (TSA), a potent HDAC inhibitor, increases *Abcd2* expression in hepatocytes only at high doses

From these results, we hypothesized that in hepatocytes only part of PBA induction of *Abcd2* can be ascribed to HDAC inhibition, and the other part to PPARα activation. To test this hypothesis, we treated rat primary hepatocytes with TSA, a specific inhibitor of HDAC at nanomolar concentrations, using different doses (0.03–3 μM) and times (24, 48, and 72 h). Expression of *Abcd2* was enhanced only at high doses (1–3 μM) of TSA, and the response was limited (×1.6 on average; Fig. 7).

Dose-dependent induction of the *Abcd2* promoter by PBA or butyrate requires only the basic promoter

The rat *Abcd2* promoter contains a CCAAT box (−53) surrounded by two putative GC boxes respectively located at −65 (GC2: CCGCCC) and −35 (GC1: GGGTGG), the three motifs being very well conserved in mouse and human (Fourcade et al., 2001). To identify the molecular mechanism of induction by PBA, COS-7 cells were transiently transfected with a plasmid construct (p277) containing the first 83 bp of the promoter (Fourcade et al., 2001) upstream from the luciferase reporter gene. Transfected cells were then exposed to different doses of PBA or butyrate for 36 h and analyzed for their luciferase activity. Under fibrate treatment, the construct was unable to mediate any induction in similar experiments (Fourcade et al., 2001). In the presence of PBA or butyrate, we observed a strong dose-dependent induction (Fig. 8 A). Similar results were obtained with the p2206 construct containing 2 kb of the

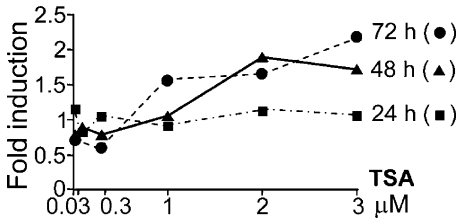

Figure 7. **TSA, a potent HDAC inhibitor, induces *Abcd2* expression.** Rat primary hepatocytes were treated with different doses of TSA and for different times. *Abcd2* expression was analyzed by RT-PCR. Results from three independent experiments are presented as fold induction of the control values (equal to 1) for untreated cells.

Published April 4, 2005

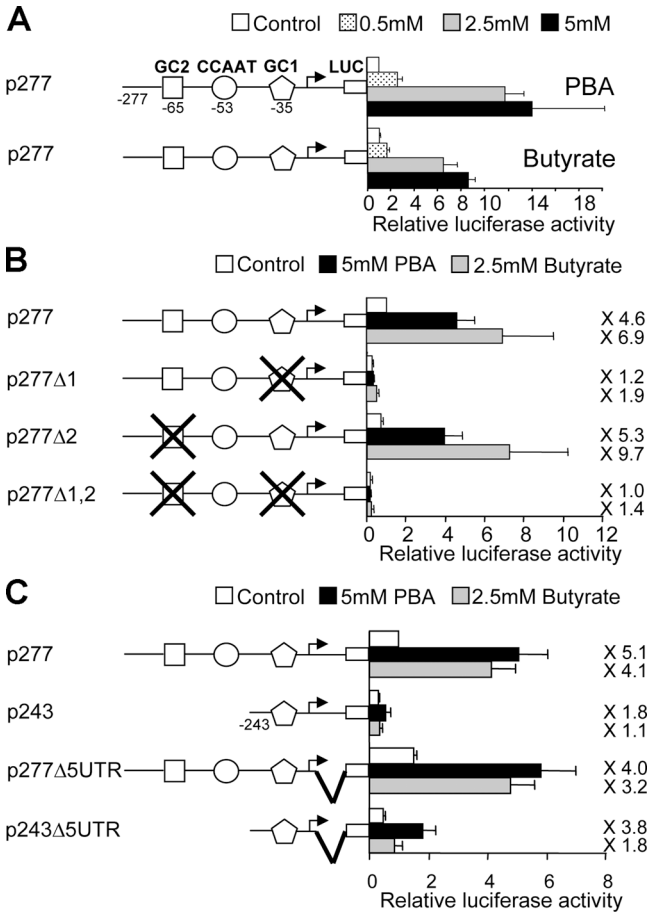

Figure 8. **The GC and CCAAT boxes control basal expression and allow full induction of the basic *Abcd2* promoter by PBA and butyrate.** COS-7 cells were cotransfected with constructs containing normal or mutated *Abcd2* promoter upstream of the luciferase reporter gene and pCMV-βGal to allow normalization of the luciferase activity. Mutations of the GC and CCAAT boxes and deletions are indicated in the schematic diagram. The cells were cultured with or without different doses of PBA or butyrate. (A) Analysis of the dose-dependent effect of PBA and butyrate. (B) Analysis of the role of the GC boxes. (C) Analysis of the role of the CCAAT box and of 5′UTR sequence. In each experiment, results are presented in relation to the luciferase activity of untreated p277-transfected cells taken equal to 1. Fold induction showing the effect of the treatment for each construct is indicated. Data are shown as means ± SD of three to six independent experiments performed in triplicate wells.

promoter (p2206) (unpublished data), indicating that the basic promoter (83 bp) is sufficient to mediate full induction of the *Abcd2* gene by PBA.

PBA-response and basal activity of the *Abcd2* promoter depends on a GC box and a CCAAT box

It has been reported that mutation or deletion of a GC box can abolish the response to butyrate (Lu and Lotan, 1999) and that HDAC1 can repress transcription by direct interaction with Sp1 and Sp3 (Doetzlhofer et al., 1999; Davie, 2003). The CCAAT box has also been demonstrated to play an important role in gene activation by HDAC inhibitors through the CCAAT-binding protein NF-Y (a trimeric association of NF-YA, NF-YB, and NF-YC) and recruitment of histone acetyl transferase (HAT) activity (McCaffrey et al., 1997; Jin and Scotto, 1998). Therefore, we further analyzed the role of the GC and CCAAT boxes in the basal activity of the *Abcd2* promoter and in the context of PBA induction.

We first compared in independent experiments the activity of p277 with the activities of p277Δ1, p277Δ2, and p277Δ1,2 (where one or both GC boxes is mutated; Fig. 8 B) or with the activities of p243 (construct devoid of 34 bp of the 5′ region containing the GC2 and CCATT boxes), p277Δ5UTR, and p243Δ5UTR (constructs devoid of 52 bp of the 5′ untranslated region) (Fig. 8 C). Mutation of the GC1 box markedly decreased the basal activity of the reporter gene (3.6- and 5.6-fold reduction for p277Δ1 and p277Δ1,2, respectively), underlining the functional importance of this GC box for basal *Abcd2* expression (Fig. 8 B). By contrast, mutation of the GC2 box caused a minor decrease in basal activity (Fig. 8 B). Deletion of the 34 bp containing the GC2 and CCATT boxes displayed a 3.2-fold decrease in the basal activity, which is more pronounced than the one observed with p277Δ2 (1.3-fold; Fig. 8 C). This observation suggests a functional role for the CCAAT box in the basal activity of the promoter. Moreover, basal activity of p277Δ5UTR was 1.5-fold higher, suggesting that some repression was mediated through the deleted 5′-UTR sequence. However, deletion of the 5′-UTR sequence in the p243 construct did not show any difference (Fig. 8 C).

Interestingly, the effect of PBA and butyrate treatment was abolished by the mutation of the GC1 box, indicating that the GC1 box is not only essential for basal activity but is also the key element of the activation by PBA or butyrate (Fig. 8 B). This capability of induction was also decreased in the p243 construct (1.8-fold induction) showing that the presence of the GC1 box is sufficient to mediate PBA induction but suggesting that the presence of the CCAAT box (which is not sufficient alone; see p277Δ1,2) is necessary to mediate full induction by PBA (Fig. 8 C). Deletion of the 5′-UTR sequences did not alter significantly the levels of induction by PBA or butyrate (Fig. 8 C).

The GC1 and CCAAT boxes bind their cognate factors, and HDAC-1 is recruited through the GC1 box

To explore the capability of the GC1 and CCAAT boxes of the *Abcd2* promoter to bind in vitro Sp1 or Sp3 and NF-Y, respectively, we first performed electrophoretic mobility shift assay (EMSA) with the corresponding radiolabeled oligonucleotides. Several DNA–protein complexes were formed in the presence of nuclear extracts from rat liver (Fig. 9 A, lanes 2 and 10). These complexes were sequence-specific because they disappeared in the presence of an excess of unlabeled homologous oligonucleotide (Fig. 9 A, lanes 3 and 11) but not in the presence of an excess of unlabeled mutated oligonucleotide (Fig. 9 A, lanes 4 and 12). To investigate if the retarded complexes result from the binding of Sp1/Sp3 and NF-Y, we performed EMSA in the presence of the specific antibodies. Incubation with the anti-Sp1 and/or anti-Sp3 antibodies resulted in specific supershifted complexes for both antibodies, indicating that the GC1 box binds Sp1 and Sp3 (Fig. 9 A, lanes 5–7). A supershift was also obtained with the anti–NF-YA antibody

Published April 4, 2005

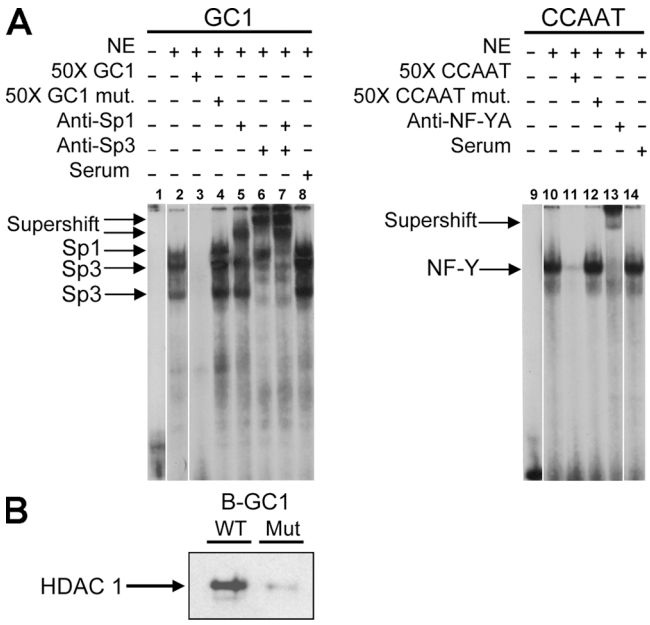

Figure 9. **Sp1/Sp3 and NF-Y bind to the GC1 and CCAAT boxes and HDAC1 is recruited through the GC1 box.** (A) EMSA was performed using the ^{32}P-labeled GC1 or CCAAT oligonucleotides with 10 μg of nuclear extracts (NE) from rat liver. Competition experiments were performed with a 50-fold molar excess of unlabeled oligonucleotides. Supershifts were performed using specific antibodies or serum for control. Retarded complexes are indicated. (B) DAPA was performed with the same nuclear extracts using the biotinylated normal or mutated GC1 oligonucleotide. Complexed proteins were resolved by SDS-PAGE and revealed by Western blot using an anti-HDAC1 antibody.

and the CCAAT probe (Fig. 9 A, lane 13), demonstrating that the CCAAT box binds NF-Y.

We further investigated by DNA affinity precipitation assay (DAPA) if the GC1 motif could be involved in the recruitment of HDAC1. We used the biotinylated oligonucleotides corresponding to the GC1 and mutated GC1 sequences used in EMSA. The presence of HDAC-1 was detected in the precipitated complexes obtained with the normal GC1 box but not with the mutated motif (Fig. 9 B). This finding indicates that HDAC-1 is recruited through interaction with the Sp1/Sp3 factors bound to the GC1 box of the *Abcd2* promoter.

Discussion

Because PBA induces peroxisome proliferation in human fibroblasts, PBA has been described as a new PP (Kemp et al., 1998). However, the fact that *AOX* and *PPAR*α expression was unchanged in these cells indicates that PBA may induce peroxisome proliferation through a mechanism not shared by classical PPs such as fibrates. To clarify this point, we studied the effects of PBA in rodent tissues and cells. Induction of the *Abcd2* and *Aox* expression and of the peroxisome proliferation in the liver of rats was moderate and occurred only after a long-term (6–9-wk) treatment with high doses of PBA. The observed alterations in the shape (phi-bodies) and in the distribution (clusters and nearly touching organelles) of peroxisomes are reminiscent of PP-induced modifications already described in rat liver (Svoboda and Azarnoff, 1966; Baumgart et al., 1989; Gorgas and Krisans, 1989; Roels, 1991) and rat hepatoma cells (Duclos et al., 1997). It has been shown that overexpression of Pex11p in rodent or human cells causes peroxisome proliferation, characterized by initial conversion of peroxisomes from spherical vesicles into elongated tubules followed by appearance of numerous small vesicular peroxisomes (Passreiter et al., 1998; Schrader et al., 1998; Li and Gould, 2002). In the liver of rodents treated with PPs, peroxisome proliferation and peroxisomal gene induction occur early. In contrast, PBA had no effect when a short-term treatment (3 d) was given to rats, suggesting a different mode of action. However, the absence of effect could result from the rapid metabolism of PBA to phenylacetate leading to ineffective PBA plasma levels. Therefore, we exposed primary hepatocytes to high concentrations of PBA for 3 d and observed induction of the peroxisomal genes and peroxisome proliferation hardly higher than in vivo, indicating that PBA is a low-potent PP. It is well known that the peroxisome proliferation in the rodent liver due to classical PPs is totally suppressed in *PPAR*α −/− mice (Lee et al., 1995). In contrast, the present work shows that the PBA-induced peroxisome proliferation is maintained in *PPAR*α −/− hepatocytes. Both PBA and classical PPs induce *Pex11*α expression in mammals (Abe and Fujiki, 1998; Abe et al., 1998; Kemp et al., 1998; Schrader et al., 1998; Depreter et al., 2002). Surprisingly, Pex11α is dispensable for peroxisome proliferation mediated by PPARα activators, whereas Pex11α is required for peroxisome proliferation in response to PBA (Li et al., 2002). Furthermore, PBA but not fibrates induces peroxisome proliferation in nonhepatic cells (where PPARα expression is lower than in hepatocytes), namely fibroblasts (Kemp et al., 1998) and C6 cells (the present study). In summary, the requirement of PPARα and Pex11α for peroxisome proliferation and the cell type where peroxisome proliferation can occur are different for PBA and classical PPs. This finding indicates that the mechanism of peroxisome proliferation in response to PBA is distinct from that used by PPs.

X-ALD is characterized by demyelination in the brain. In the context of a pharmacological therapy based on the partial functional redundancy of the peroxisomal transporter ALDRP there is considerable interest to explore the effects of PBA in the brain. PBA is likely to cross the blood–brain barrier because it is effective in lowering VLCFA levels in the brain of *Abcd1* −/− mice (Kemp et al., 1998). Indeed, modifications at the peroxisomal level were detected in ependymal cells of the fourth ventricle in PBA-treated rats. Unfortunately, no induction in peroxisomal gene expression was seen in the brain examined as a whole. Nevertheless, the induction of *Abcd2* that we observed in PBA-treated glial cells might account for the restoration of VLCFA peroxisomal β-oxidation in the brain of PBA-treated *Abcd1* −/− mice described by Kemp et al. (1998).

The knowledge of the molecular mechanism underlying the PBA effects on *Abcd2* expression may provide leads in searching for new drugs for X-ALD therapy. We compared PBA with ciprofibrate or fenofibrate (classical powerful PPs) and with butyrate (an HDAC inhibitor) for their effects on peroxisomal gene expression in hepatic or nonhepatic cell cul-

Published April 4, 2005

tures. First, in fibroblasts and glial cells, fibrate was unable to induce *Abcd2*, suggesting that a cofactor of PPARα is missing or that the PPARα level is too low (Lemberger et al., 1996). In contrast, *Abcd2* was activated by PBA or butyrate in these cells, and we demonstrated that the activation is PPARα independent. Furthermore, in hepatocytes, *Abcd2* and *Aox* were induced by PBA, butyrate, and ciprofibrate, with some differences for the three compounds. We had observed that the induction of these genes by fibrate was completely abolished in the liver of *PPARα −/−* mice (Fourcade et al., 2001). Here, we show that PBA induction of *Abcd2* is only reduced in *PPARα −/−* hepatocytes, suggesting that part of the induction is PPARα dependent and that the other part would originate in the same mechanism underlying the activation of *Abcd2* by PBA and butyrate in nonhepatic cells. Similar conclusions can be drawn from our experiments on TSA-treated hepatocytes. Altogether, the results indicate that PBA and butyrate would mainly activate *Abcd2* by HDAC inhibition, whereas in hepatocytes an extra induction occurs through PPARα. This PPARα-dependent induction is in agreement with the relatively high level of PPARα in hepatocytes and the capacity of PBA to bind PPARα (Liu et al., 2002).

HDAC is known to interact with the transcription complex and the chromatin in a DNA region surrounding the transcription start, resulting in enhanced transcription rate (Nakayama and Takami, 2001). A basic promoter should be sufficient so that HDAC activity can take place. Support for this concept was obtained from our work demonstrating that the first 49 bp of the *Abcd2* promoter enable PBA and butyrate to activate a reporter gene and that the first 83 bp enable full induction. In transient transfection experiments, we demonstrated that the PBA-dependent induction and the basal level of expression of *Abcd2* depends on the GC1 and CCAAT boxes. Both motifs were shown to bind their cognate factors Sp1/Sp3 and NF-Y, respectively, and we demonstrated that HDAC-1 is recruited through the GC1 box in agreement with previous papers (Doetzlhofer et al., 1999). Expression of *Abcd2* probably results from the balanced action of HAT and HDAC activities. The CCAAT box, which binds NF-Y, likely allows HAT activity to be recruited and to antagonize the effect of HDAC-1 (Jin and Scotto, 1998). The recruitment of HAT activity may depend not only on NF-Y but also on Sp1, as it has already been described for the promoter of *HDAC1* for instance (Schuettengruber et al., 2003). It would explain why basal activity of the *Abcd2* promoter is reduced when the GC1 box is mutated, whereas it might have been expected that this mutation results in enhanced promoter activity because HDAC1 is not recruited anymore. This would also be in agreement with the up-regulation of HAT activity by physical interaction with Sp1 (Soutoglou et al., 2001). Besides, it was shown that the effect of PBA on *Abcd2* expression strictly depends on the presence of the GC1 box and consequently on the recruitment of HDAC1. However, full induction has been observed only when the CCAAT box is present, suggesting a synergistic effect of the two motifs. HDAC inhibition could increase recruitment of HAT to the CCAAT box as it has already been hypothesized for the *TβRII* gene (Park et al., 2002). Further work would be necessary to define more precisely the complexes containing HAT and HDAC activities and to show if other HDACs of the class I (Marks et al., 2003) can be involved in *Abcd2* expression.

We have shown that fibrates induce *Abcd2* expression in the liver of rodents in a PPARα-dependent way but not in the brain (Albet et al., 1997; Berger et al., 1999; Fourcade et al., 2001). It was believed that the absence of effects in brain was due to their inability to cross the blood–brain barrier. Actually, the present study revealed that fibrate, a powerful PP, has no effect in glial cells and fibroblasts. This lack of effect suggests that any drug, which requires PPARα, is likely to be ineffective in inducing *Abcd2* expression and in restoring VLCFA β-oxidation in the brain of *Abcd1 −/−* mice or X-ALD patients. PBA might be a better candidate for a pharmacological therapy of X-ALD because we showed that it can induce *Abcd2* expression in glial cells and cause peroxisome proliferation. An increased number of peroxisomes could contribute to improve VLCFA metabolism in X-ALD patients. However, it is still unclear whether or not the correction of VLCFA levels observed in the brain of *Abcd1 −/−* mice after PBA treatment (Kemp et al., 1998) results from enhanced *Abcd2* expression. A high effective dose and a decreased response in long-term studies are two stumbling blocks in using PBA for a therapy (McGuinness et al., 2001). In an effort to identify nontoxic agents effective at a low dose and with a long-term effect, we searched for the mechanism of pharmacological induction of *Abcd2* by PBA. We show that PBA acts at the peroxisomal level probably by inhibiting HDAC activity, like butyrate, suggesting that other HDAC inhibitors should be tested. Two compounds structurally related to PBA (styrylacetate and benzyloxyacetate) were shown to be as efficient as PBA in increasing VLCFA peroxisomal β-oxidation in *Abcd1 −/−* mice fibroblasts (McGuinness et al., 2001). It would be of great interest to assess the potential of these two analogues on *Abcd2* expression in oligodendrocytes, the myelin-forming cells.

There is a priori concern regarding the use of HDAC inhibitors because of their nonspecific effects on gene expression, but in clinical trials several HDAC inhibitors appeared to be well tolerated (Marks et al., 2003). Investigation of pharmacological agents acting through another molecular mechanism should also be considered. A more comprehensive understanding of the expression of the *Abcd2* gene is needed to identify other drugs for the development of an effective pharmacological therapy.

Materials and methods

Animals and treatments

Male Wistar rats of ~200 g (Charles River Laboratories) were kept at 22°C with equal periods of darkness and light and had free access to water and food. PBA (Sigma-Aldrich) was delivered to rats ($n = 6$) in their chow (7.5 g/kg) and water (10 g/L) corresponding to dose ×1 for 6–7 wk. A higher dose of PBA (dose ×1.5), corresponding to 11.3 g/kg of chow and 15 g/L of water, was also given to rats ($n = 4$) for 6–9 wk. The daily PBA uptake was estimated to be ~0.4–0.5 g per day per rat. The daily increase in body weight was significantly lower in treated animals (2.9 g) than in controls (5.3 g). Moreover, rats ($n = 3$) received 720 mg PBA per kilogram of body weight per day for 3 d via i.p. injections with an aqueous solution of PBA (40 mg/ml) twice a day. They were killed 12 h after the last injection.

Rats (400 g) were anesthetized with pentobarbital (60 mg/kg, i.p.). Drugs were administered at a delivery rate of 100 μl/h through a cannula stereotaxically implanted into the fourth ventricle. Rats received ei-

Published April 4, 2005

ther 2 mg of sodium ciprofibrate ($n = 4$) or 20 mg of PBA ($n = 4$) dissolved in a total volume of 100 μl of sterile water (pH adjusted at 7.3). Control rats ($n = 4$) received equiosmolar amounts of NaCl (100 μl). The rats were killed 48 h after injection.

Cell culture

Rat primary hepatocytes were prepared as described previously (Fourcade et al., 2001). Mouse primary hepatocytes were obtained according to Maslansky and Williams (1982), and a transfer on a cushion of Percoll yielded 90% viability. Differentiated CG4 rat glial cells, pure primary astocytes, and mixed primary culture of oligodendrocytes and astrocytes were obtained and cultured as described previously (Fourcade et al., 2003). Embryonic fibroblasts were prepared from 14-d-old fetuses of *PPARα* +/+ and −/− mice as described previously (Doetschman et al., 1985) and cultured in DME supplemented with 10% FCS. COS-7 cells were grown in DME supplemented with 10% FCS in the absence of antibiotics.

Northern blot analysis

Total RNA was extracted from rat tissues using a standard protocol. The GenElute Mammalian Total RNA kit (Sigma-Aldrich) was used to prepare RNA from cultured cells. Membranes containing 20 μg per lane of RNA were hybridized with α-[^{32}P]–labeled cDNA probe as described previously (Albet et al., 2001).

Semiquantitative RT-PCR

Total RNA was reverse-transcribed by random priming. To study *Aox* expression, PCR was performed as described previously (Fourcade et al., 2001). *Abcd2* cDNA was amplified using the forward (F) and reverse (R) primers 5′-GTGTATGCCACTGCTAAAG-3′ and 5′-TCAGCTCCAGAGGCCAGT-3′, respectively, for 27 cycles (30 cycles when RNA was extracted from primary hepatocytes) and 56°C as the annealing temperature. Amplification of *36b4* cDNA was performed using the primers 5′-AAYGTGGGCTCCAAGCAGATG-3′ (F) and 5′-GAGATGTTCAYCATGTTCAGCAG-3′ (R), for 17 cycles and 60°C as the annealing temperature. PCR products were analyzed on agarose gels and quantified by digital imaging, and the relative abundance of mRNA was determined by comparison with *36b4* mRNA level.

Microscopy

Rats were anesthetized and the tissues were fixed through intracardial perfusion with fixative. Peroxisomes were visualized by staining for catalase activity with DAB (Roels et al., 1995). Immunolocalization of AOX (antibody provided by A. Völkl, University of Heidelberg, Heidelberg, Germany) or catalase for LM or EM was performed according to Espeel and Van Limbergen (1995). LM images were photographed on film on an Aristoplan microscope (Leitz) using 63×, 1.4 NA, and 40×, 0.75 NA, oil immersion lenses (Leitz) for bright field and phase contrast and on an inverted microscope (model DMIRB; Leica) using a Leica PL Apo 63×, 1.32–0.6 NA oil immersion lens, and the Leica MPS-60 photoautomat for immunofluorescence. On randomly taken LM micrographs, the number of peroxisomes per cellular area was determined by counting catalase-stained peroxisomes and measuring cellular area excluding blood vessels with a semi-automatic image analysis system (Mini-Mop; Kontron). EM images were photographed on a transmission electron microscope (model JEM-100B; JEOL). Morphometry of peroxisomes was performed on random EM micrographs as described previously (Kerckaert et al., 1995). Gold particles per peroxisome were counted and organelle area was measured with the Mini-Mop apparatus. A nonparametric ranking test (Mann-Whitney) was used for statistical analysis of microscopy data. For publication, micrographs were scanned and imported into Adobe Photoshop.

Plasmid constructs

The first 83 bp of the rat *Abcd2* promoter (relative to the transcription start located at −194 from the translation start site) were cloned directionally into pGL3-Basic (Promega) to yield p277 as described previously (Fourcade et al., 2001). The p243 construct was prepared as p277 except that the inserted PCR fragment was amplified using forward primer 5′-ttagatcTGAGAAGCCTGGG-3′ containing a BglII site (underlined). The plasmids p277Δ5UTR and p243Δ5UTR were built as p277 and p243 using the reverse primer 5′-CTGaAGCTTGCTCAAACTCCACCGC-3′ (the HindIII site is underlined). The constructs were verified by sequencing with GL primer 2 (Promega). p277Δ1, p277Δ2, and p277Δ1,2 were obtained from the p277 plasmid by site-directed mutagenesis with the Quickchange site directed mutagenesis kit (Stratagene) according to the manufacturer's protocol using pairs of complementary oligonucleotides corresponding to the following sequences: GC1 mut, 5′-CCAATGAGAAGCCTGGG**GTTTG**-**G**AGCCTGGCCAGC-3′; GC2 MUT, 5′-GGAGTCCAGGGTCTCA**AAGCCC**ACGCAGCCAATGAG-3′ (the GC boxes are shown in bold and mutations are underlined).

Transient transfection experiments

COS-7 cells were transfected using Exgen 500 liposome (Euromedex) with 100 ng of pCMV-βGal (CLONTECH Laboratories, Inc.), 650 ng of pGLUC (a modified pGL2 vector) or empty pGL3-Basic or construct, and 250 ng of empty pSG5 (Stratagene) as previously described (Fourcade et al., 2001). The transfected cells were incubated with or without PBA or butyrate for 36 h and assayed for luciferase activity (Fourcade et al., 2001). Luciferase activity was corrected for transfection efficiency using β-galactosidase activity.

EMSA

The following pairs of oligonucleotides used as probes or competitors in EMSA were designed from the rat *ABCD2* promoter: GC1 (F, 5′-agcttCCTGGG**GGGTGG**AGCCTGG-3′; R, 5′-gatcCCAGGCT**CCACCC**CCCAGGa-3′), GC1 mut (F, 5′-agcttCCTGGG**GTTTGG**AGCCTGG-3′; R, 5′-gatcCCAGGCT**CCAAAC**CCCAGGa-3′), CCAAT (F, 5′-agcttCCACGCAG**CCAAT**GAGAAGCCg-3′; R, 5′-gatccGGCTTCTC**ATTGG**CTGCGTGGa-3′), CCAAT mut (F, 5′-agcttCCACGCCC**CGTAT**TTGAAGCCg-3′; R, 5′-gatccGGCTTCAA**ATACG**GGGCGTGGa-3′) (GC1 and CCAAT boxes are shown in bold; mutations are underlined; lower case letters are modified nucleotides from the original sequence to create compatible ends for cloning and fill-in labeling). Labeling of probes and binding experiments in the presence of 10 μg of nuclear extracts from rat liver were performed as described previously (Fourcade et al., 2001). Competition and supershift experiments were performed by adding a 50-fold molar excess of unlabeled oligonucleotides or antibodies (Santa Cruz Biotechnology, Inc.; anti-Sp1, anti-Sp3, and anti–NF-YA) or a rabbit serum (control) 30 min before the addition of the radiolabeled probes. The samples were analyzed by electrophoresis at 4°C on 4% polyacrylamide gels in 0.5 × TBE (45 mM Tris-borate and 1 mM EDTA).

DAPA

Complementary oligonucleotides corresponding to the normal (5′-TGAGAAGCCTGGG**GGGTGG**AGCCTGGCCAG-3′) or mutated (5′-TGAGAAGCCTGGG**GTTTGG**AGCCTGGCCAG-3′) GC1 box were synthesized, biotinylated at their 5′ termini, and annealed. The DAPA was performed in a total volume of 400 μl by mixing 4 μg of biotinylated DNA probe with 50 μg of nuclear extracts from rat liver in binding buffer (20 mM Hepes, pH 7.9, 10% glycerol, 50 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 10 μM ZnCl2, 1 mM DTT, and 0.25% Triton X-100). After a 30-min preincubation on ice, 50 μl of streptavidin-agarose beads (Novagen) were added and incubated for 2 h under agitation. Beads were collected by a brief centrifugation and washed twice in binding buffer. Proteins were eluted in 15 μl of SDS-PAGE loading buffer by heating for 5 min at 95°C. After centrifugation, the supernatant was loaded on SDS-PAGE and Western blot analysis was performed using anti-HDAC1 antibody (Santa Cruz Biotechnology, Inc.).

We dedicate this paper to the memory of Professor Maurice Bugaut, who initiated this project and who died an untimely death while the manuscript was being revised.

We thank Professor Alfred Völkl for acyl-CoA oxidase antibody. We also thank Betty De Prest, Dominique Jacobus, Hubert Stevens, Simonne Van Hulle, and Guido Van Limbergen (all from Ghent University) for their technical assistance.

This work was supported by grants from the European Association against Leukodystrophies (ELA) and the Regional Council of Burgundy. S. Fourcade and S. Leclercq were supported by a fellowship from ELA. S. Fourcade was also supported by a fellowship from Fondation pour la Recherche Médicale.

Submitted: 7 January 2005
Accepted: 3 March 2005

References

Abe, I., and Y. Fujiki. 1998. cDNA cloning and characterization of a constitutively expressed isoform of the human peroxin Pex11p. *Biochem. Biophys. Res. Commun.* 252:529–533.

Abe, I., K. Okumoto, S. Tamura, and Y. Fujiki. 1998. Clofibrate-inducible, 28-kDa peroxisomal integral membrane protein is encoded by PEX11. *FEBS Lett.* 431:468–472.

Albet, S., C. Causeret, M. Bentejac, J.L. Mandel, P. Aubourg, and M. Bugaut.

1997. Fenofibrate differently alters expression of genes encoding ATP-binding transporter proteins of the peroxisomal membrane. *FEBS Lett.* 405:394–397.

Albet, S., M. Bentejac, S. Savary, C. Gondcaille, A. Netik, J. Berger, C. Szpirer, N. Troffer-Charlier, and M. Bugaut. 2001. Rat adrenoleukodystrophy-related (ALDR) gene: full-length cDNA sequence and new insight in expression. *Biochim. Biophys. Acta.* 1517:257–269.

Baumgart, E., A. Volkl, T. Hashimoto, and H.D. Fahimi. 1989. Biogenesis of peroxisomes: immunocytochemical investigation of peroxisomal membrane proteins in proliferating rat liver peroxisomes and in catalase-negative membrane loops. *J. Cell Biol.* 108:2221–2231.

Berger, J., S. Albet, M. Bentejac, A. Netik, A. Holzinger, A.A. Roscher, M. Bugaut, and S. Forss-Petter. 1999. The four murine peroxisomal ABC-transporter genes differ in constitutive, inducible and developmental expression. *Eur. J. Biochem.* 265:719–727.

Braiterman, L.T., S. Zheng, P.A. Watkins, M.T. Geraghty, G. Johnson, M.C. McGuinness, A.B. Moser, and K.D. Smith. 1998. Suppression of peroxisomal membrane protein defects by peroxisomal ATP binding cassette (ABC) proteins. *Hum. Mol. Genet.* 7:239–247.

Davie, J.R. 2003. Inhibition of histone deacetylase activity by butyrate. *J. Nutr.* 133:2485S–2493S.

Depreter, M., J. Vandesompele, M. Espeel, F. Speleman, and F. Roels. 2002. Modulation of the peroxisomal gene expression pattern by dehydroepiandrosterone and vitamin D: therapeutic implications. *J. Endocrinol.* 175:779–792.

Doetschman, T.C., H. Eistetter, M. Katz, W. Schmidt, and R. Kemler. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* 87:27–45.

Doetzlhofer, A., H. Rotheneder, G. Lagger, M. Koranda, V. Kurtev, G. Brosch, E. Wintersberger, and C. Seiser. 1999. Histone deacetylase 1 can repress transcription by binding to Sp1. *Mol. Cell. Biol.* 19:5504–5511.

Duclos, S., J. Bride, L.C. Ramirez, and P. Bournot. 1997. Peroxisome proliferation and beta-oxidation in Fao and MH1C1 rat hepatoma cells, HepG2 human hepatoblastoma cells and cultured human hepatocytes: effect of ciprofibrate. *Eur. J. Cell Biol.* 72:314–323.

Espeel, M., and G. Van Limbergen. 1995. Immunocytochemical localization of peroxisomal proteins in human liver and kidney. *J. Inherit. Metab. Dis.* 18:135–154.

Flavigny, E., A. Sanhaj, P. Aubourg, and N. Cartier. 1999. Retroviral-mediated adrenoleukodystrophy-related gene transfer corrects very long chain fatty acid metabolism in adrenoleukodystrophy fibroblasts: implications for therapy. *FEBS Lett.* 448:261–264.

Fourcade, S., S. Savary, S. Albet, D. Gauthe, C. Gondcaille, T. Pineau, J. Bellenger, M. Bentejac, A. Holzinger, J. Berger, and M. Bugaut. 2001. Fibrate induction of the adrenoleukodystrophy-related gene (ABCD2): promoter analysis and role of the peroxisome proliferator-activated receptor PPARalpha. *Eur. J. Biochem.* 268:3490–3500.

Fourcade, S., S. Savary, C. Gondcaille, J. Berger, A. Netik, F. Cadepond, M. ElEtr, B. Molzer, and M. Bugaut. 2003. Thyroid hormone induction of the adrenoleukodystrophy-related gene (ABCD2). *Mol. Pharmacol.* 63:1296–1303.

Gartner, J., G. Jimenez-Sanchez, P. Roerig, and D. Valle. 1998. Genomic organization of the 70-kDa peroxisomal membrane protein gene (PXMP1). *Genomics.* 48:203–208.

Gorgas, K., and S.K. Krisans. 1989. Zonal heterogeneity of peroxisome proliferation and morphology in rat liver after gemfibrozil treatment. *J. Lipid Res.* 30:1859–1875.

Holzinger, A., S. Kammerer, and A.A. Roscher. 1997. Primary structure of human PMP69, a putative peroxisomal ABC-transporter. *Biochem. Biophys. Res. Commun.* 237:152–157.

Jin, S., and K.W. Scotto. 1998. Transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is mediated by NF-Y. *Mol. Cell. Biol.* 18:4377–4384.

Kamijo, K., S. Taketani, S. Yokota, T. Osumi, and T. Hashimoto. 1990. The 70-kDa peroxisomal membrane protein is a member of the Mdr (P-glycoprotein)-related ATP-binding protein superfamily. *J. Biol. Chem.* 265:4534–4540.

Kemp, S., H.M. Wei, J.F. Lu, L.T. Braiterman, M.C. McGuinness, A.B. Moser, P.A. Watkins, and K.D. Smith. 1998. Gene redundancy and pharmacological gene therapy: implications for X-linked adrenoleukodystrophy. *Nat. Med.* 4:1261–1268.

Kerckaert, I., D. De Craemer, and G. Van Limbergen. 1995. Practical guide for morphometry of human peroxisomes on electron micrographs. *J. Inherit. Metab. Dis.* 18:172–180.

Lea, M.A., and V.M. Randolph. 1998. Induction of reporter gene expression by inhibitors of histone deacetylase. *Anticancer Res.* 18:2717–2722.

Lee, S.S.-T., T. Pineau, J. Drago, E.J. Lee, J.W. Owens, D.L. Kroetz, P.M. Fernandez-Salguero, H. Westphal, and F.G. Gonzalez. 1995. Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol. Cell. Biol.* 15:3012–3022.

Lemberger, T., O. Braissant, C. Juge-Aubry, H. Keller, R. Saladin, B. Staels, J. Auwerx, A.G. Burger, C.A. Meier, and W. Wahli. 1996. PPAR tissue distribution and interactions with other hormone-signaling pathways. *Ann. NY Acad. Sci.* 804:231–251.

Li, X., and S.J. Gould. 2002. PEX11 promotes peroxisome division independently of peroxisome metabolism. *J. Cell Biol.* 156:643–651.

Li, X., E. Baumgart, G.-X. Dong, J.C. Morrell, G. Jimenez-Sanchez, D. Valle, K.D. Smith, and S.J. Gould. 2002. PEX11α is required for peroxisome proliferation in response to 4-phenylbutyrate but is dispensable for peroxisome proliferator-activated receptor α-mediated peroxisome proliferation. *Mol. Cell. Biol.* 22:8226–8240.

Liu, N., W. Qiang, X. Kuang, P. Thuillieer, W.S. Lynn, and P.K.Y. Wong. 2002. The peroxisome proliferator phenylbutyric acid (PBA) protects astrocytes from ts1 MoMuLV-induced oxidative cell death. *J. Neurovirol.* 8:318–325.

Lombard-Platet, G., S. Savary, C.O. Sarde, J.L. Mandel, and G. Chimini. 1996. A close relative of the adrenoleukodystrophy (ALD) gene codes for a peroxisomal protein with a specific expression pattern. *Proc. Natl. Acad. Sci. USA.* 93:1265–1269.

Lu, Y., and R. Lotan. 1999. Transcriptional regulation by butyrate of mouse galectin-1 gene in embryonal carcinoma cells. *Biochim. Biophys. Acta.* 1444:85–91.

Marks, P.A., T. Miller, and V.M. Richon. 2003. Histone deacetylases. *Curr. Opin. Pharmacol.* 3:344–351.

Maslansky, C.J., and G.M. Williams. 1982. Primary cultures and the levels of cytochrome P450 in hepatocytes from mouse, rat, hamster and rabbit liver. *In Vitro.* 18:683–693.

McCaffrey, P.G., D.A. Newsome, E. Fibach, M. Yoshida, and M.S. Su. 1997. Induction of gamma-globin by histone deacetylase inhibitors. *Blood.* 90:2075–2083.

McGuinness, M.C., H.P. Zhang, and K.D. Smith. 2001. Evaluation of pharmacological induction of fatty acid beta-oxidation in X-linked adrenoleukodystrophy. *Mol. Genet. Metab.* 74:256–263.

Moser, H.W., K.D. Smith, P.A. Watkins, J. Powers, and A.B. Moser. 2001. X-linked adrenoleukodystrophy. *In* The Metabolic & Molecular Bases of Inherited Disease, 8th edition. R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, editors. McGraw-Hill Inc., New York. 3257–3301.

Mosser, J., A.M. Douar, C.O. Sarde, P. Kioschis, R. Feil, H. Moser, A.M. Poustka, J.L. Mandel, and P. Aubourg. 1993. Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. *Nature.* 361:726–730.

Motojima, K. 1997. Peroxisome proliferator-activated receptor (PPAR)-dependent and -independent transcriptional modulation of several non-peroxisomal genes by peroxisome proliferators. *Biochimie.* 79:101–106.

Nakayama, T., and Y. Takami. 2001. Participation of histones and histone-modifying enzymes in cell functions through alterations in chromatin structure. *J. Biochem. (Tokyo).* 129:491–499.

Netik, A., S. Forss-Petter, A. Holzinger, B. Molzer, G. Unterrainer, and J. Berger. 1999. Adrenoleukodystrophy-related protein can compensate functionally for adrenoleukodystrophy protein deficiency (X-ALD): implications for therapy. *Hum. Mol. Genet.* 8:907–913.

Park, S.H, S.R. Lee, B.C. Kim, E.A. Cho, S.P. Patel, H.B. Kang, E.A. Sausville, O. Nakanishi, J.B. Trepel, B.I. Lee, S.J. Kim. 2002. Transcriptional regulation of the transforming growth factor beta type II receptor gene by histone acetyltransferase and deacetylase is mediated by NF-Y in human breast cancer cells. *J. Biol. Chem.* 15:277:5168-5174.

Passreiter, M., M. Anton, D. Lay, R. Frank, C. Harter, F.T. Wieland, K. Gorgas, and W.W. Just. 1998. Peroxisome biogenesis: involvement of ARF and coatomer. *J. Cell Biol.* 141:373–383.

Pineau, T., W.R. Hudgins, L. Liu, L.C. Chen, T. Sher, F.J. Gonzalez, and D. Samid. 1996. Activation of a human peroxisome proliferator-activated receptor by the antitumor agent phenylacetate and its analogs. *Biochem. Pharmacol.* 52:659–667.

Pujol, A., I. Ferrer, C. Camps, E. Metzger, C. Hindelang, N. Callizot, M. Ruiz, T. Pampols, M. Giros, and J.L. Mandel. 2004. Functional overlap between ABCD1 (ALD) and ABCD2 (ALDR) transporters: a therapeutic target for X-adrenoleukodystrophy. *Hum. Mol. Genet.* 13:2997–3006.

Roels, F. 1991. Peroxisomes: A Personal Account. VUB Press, Brussels. 151 pp.

Roels, F., B. De Prest, and G. De Pestel. 1995. Liver and chorion cytochemistry. *J. Inherit. Metab. Dis.* 18:155–171.

Schrader, M., B.E. Reuber, J.C. Morrell, G. Jimenez-Sanchez, C. Obie, T.A. Stroh, D. Valle, T.A. Schroer, and S.J. Gould. 1998. Expression of PEX11beta mediates peroxisome proliferation in the absence of extracellular stimuli. *J. Biol. Chem.* 273:29607–29614.

Published April 4, 2005

Schuettengruber, B., E. Simboek, H. Khier, and C. Seiser. 2003. Autoregulation of mouse histone deacetylase 1 expression. *Mol. Cell. Biol.* 23:6993–7004.

Soutoglou, E., B. Viollet, M. Vaxillaire, M. Yaniv, M. Pontoglio, and I. Talianidis. 2001. Transcription factor-dependent regulation of CBP and P/CAF histone acetyltransferase activity. *EMBO J.* 20:1984–1992.

Svoboda, D.J., and D.L. Azarnoff. 1966. Response of hepatic microbodies to a hypolipidemic agent, ethyl chlorphenoxyisobutyrate (CPIB). *J. Cell Biol.* 30:442–450.

Waddell, W.J., C. Marlowe, M.S. Rao, and J.K. Reddy. 1989. In vivo distribution of a carcinogenic hepatic peroxisome proliferator: whole-body autoradiography of [14C]ciprofibrate in the mouse. *Carcinogenesis.* 10:221–223.

Warrell, R.P.J., L.Z. He, V. Richon, E. Calleja, and P.P. Pandolfi. 1998. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *J. Natl. Cancer Inst.* 90:1621–1625.